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Arginase

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Chapter 3

Heparin normalizes airway hyperresponsiveness and NO-deficiency

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Summary

It has been established that polycations cause airway hyperresponsiveness (AHR) to methacholine by inducing a deficiency of constitutive nitric oxide synthase (cNOS)-derived bronchodilating nitric oxide (NO). Since a deficiency of cNOS-derived NO also contributes to allergen-induced AHR after the early asthmatic reaction (EAR) and since this AHR is associated with the release of polycationic proteins from infiltrated eosinophils in the airways, we hypothesized that endogenous polycations underlie or at least contribute to the allergen-induced NO deficiency and AHR. Using a guinea pig model of allergic asthma, we addressed this hypothesis by examining the effect of the polyanion heparin, acting as a polycation antagonist, on the responsiveness to methacholine of isolated perfused tracheae from unchallenged control animals and from animals 6 h after ovalbumin challenge, *i.e*. after the EAR. A 2.0-fold AHR ($P<0.001$) to intraluminal administration of methacholine was observed in airways from allergen-challenged animals compared to control. Incubation of these airways with 250 u ml^{-1} heparin completely normalized the observed hyperresponsiveness ($P<0.001$), whereas the responsiveness to methacholine of airways from unchallenged control animals was not affected. The effect of heparin on airways from allergen-challenged guinea pigs was dose-dependently (0.1 mM and 1.0 mM) reversed by the NOS-inhibitor L-NAME ($P<0.01$). These results indicate that endogenous (presumably eosinophil-derived) polycations are involved in allergen-induced NO deficiency and AHR after the EAR, probably by inhibition of L-arginine transport.

Introduction

It has been well established that a deficiency of epithelial constitutive nitric oxide synthase (cNOS)-derived nitric oxide (NO) contributes to allergen-induced airway hyperresponsiveness (AHR) in both guinea pigs [1,2] and asthmatic patients [3]. In airways from allergen-challenged guinea pigs it was demonstrated that a reduced availability of L-arginine is involved in the observed NO deficiency and subsequent AHR after the early asthmatic reaction (EAR) [2]. Since the intracellular L-arginine concentration is under control of specific cationic amino acid-transporter systems [4], one of the mechanisms leading to a reduced availability of L-arginine after the EAR could be reduced cellular uptake of this amino acid.

Interestingly, it has been demonstrated that polycations like major basic protein (MBP), as well as its analogue poly-L-arginine, inhibit L-arginine uptake through cationic amino acid (y^+) transporters and decrease NO synthesis in rat alveolar macrophages and tracheal epithelial cells [5]. Polycation-induced inhibition of L-arginine uptake in these cells was prevented by the polyanion heparin, acting as a polycation antagonist [5]. The functional relevance of this finding was indicated by our previous study, demonstrating that poly-L-arginine causes AHR to methacholine in a perfused guinea pig tracheal tube preparation by inducing a deficiency of cNOS-

derived NO in response to this agonist [6]. Accordingly, the poly-L-arginine-induced NO deficiency and AHR were reversed by heparin [6].

The development of allergen-induced AHR after the EAR in guinea pigs is associated with the release of polycationic proteins by infiltrated eosinophils in the airways [7]. In asthmatic patients, influx and activation of eosinophils are observed as early as 3 h after allergen-challenge [8]. Furthermore, elevated levels of MBP have been demonstrated in the bronchoalveolar lavage fluid of asthmatic patients, which were correlated with the severity of AHR [9]. MBP-induced dysfunction of the airway epithelium has been implicated in the development of AHR in allergic asthma [10]. *In vitro*, it has been demonstrated that MBP is detrimental to airway epithelial cells and induces pathological alterations comparable to those observed in asthmatic patients [11]. Furthermore, intratracheal instillation or inhalation of MBP or synthetic polycations such as poly-L-arginine and poly-L-lysine caused increased airway responsiveness to methacholine in rats [12,13] and guinea pigs [14], without overt epithelial damage [12,13]. In addition, it was demonstrated that heparin suppressed polycation-induced AHR, indicating that cation-anion interactions may modulate airway responsiveness [12,14].

In the present study, we hypothesized that endogenous polycations like eosinophil-derived MBP contribute to the allergen-induced NO deficiency and AHR. This hypothesis was investigated by measuring the effect of heparin on methacholine-induced airway constriction and NO deficiency in perfused tracheal preparations from unchallenged control guinea pigs and from animals 6 h after ovalbumin-challenge, *i.e.* after the EAR, in the absence and presence of the NOS-inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME).

Methods

Animals

Outbred specified pathogen free guinea pigs (Harlan, Heathfield, United Kingdom), weighing 700 – 900 g, were used in this study. Animals were actively IgE-sensitized to ovalbumin at age of 4 weeks as previously described [15]. In short, 0.5 ml of an allergen solution containing 100 μg ml^{-1} ovalbumin and 100 mg ml^{-1} $Al(OH)_3$ in saline was injected intraperitoneally, while another 0.5 ml was divided over seven intracutaneous injection sites in the proximity of lymph nodes in the paws, lumbar regions and the neck. The animals were used experimentally 4 to 8 weeks after sensitization. The animals were group-housed in individual cages in climate controlled animal quarters and given water and food *ad libitum*, while a 12-h on/ 12-h off light cycle was maintained. All protocols described in this study were approved by the University of Groningen Committee for Animal Experimentation.

Allergen provocation

Ovalbumin provocations were performed by inhalation of aerosolized solutions. The provocations were performed in a specially designed animal cage, in which the guinea pigs could move freely [16]. The volume of the cage was 9 l, which ensured fast replacement of the air inside the cage with aerosol and *vice versa*. A DeVilbiss nebulizer (type 646, DeVilbiss, Somerset, PA, USA) driven by an airflow of 8 l min^{-1} provided the aerosol required, with an output of 0.33 ml min^{-1}. Allergen provocations were performed by inhalation of an aerosol concentration of 0.5 mg ml^{-1} ovalbumin in saline. Allergen inhalations were discontinued when the first signs of respiratory distress were observed. Anti-histamines were not needed to prevent the development of anaphylactic shock. Previous studies measuring pleural pressure changes in ovalbumin sensitized, permanently instrumented, unrestrained guinea pigs have indicated that the allergen-induced EAR induced by this procedure is maximal within 20 min and lasts for up to 5 h [16,17]. Six hours after ovalbumin challenge, the guinea pigs were sacrificed for tracheal perfusion experiments or bronchoalveolar lavage (BAL). Unchallenged or saline challenged animals, respectively, served as controls.

Tracheal perfusion

The animals were killed by a sharp blow on the head and exsanguinated. The tracheae were rapidly removed and placed in Krebs-Henseleit (KH) solution (37°C) of the following composition (mM): NaCl 117.50, KCl 5.60, $MgSO_4$ 1.18, $CaCl_2$ 2.50, NaH_2PO_4 1.28, $NaHCO_3$ 25.00, D-glucose 5.50; gassed with 5% CO_2 and 95% O_2; pH 7.4. The tracheae were prepared free of serosal connective tissue and cut into two halves of approximately 17 mm before mounting in a perfusion setup, as described previously [1]. To this aim, the tracheal preparations were attached at each end to stainless steel perfusion tubes fixed in a Delrin perfusion holder. The holder with the trachea was then placed in a water-jacketed organ bath (37°C) containing 20 ml of gassed KH (the serosal or extraluminal (EL) compartment). The lumen was perfused with recirculating KH from a separate 20 ml bath (mucosal or intraluminal (IL) compartment) at a constant flow rate of 12 ml min^{-1}. Two axially centred side-hole catheters connected with pressure transducers (TC-XX,Viggo-Spectramed B.V., Bilthoven, The Netherlands) were situated at the distal and proximal ends of the trachealis to measure hydrostatic pressures (P_{outlet} and P_{inlet}, respectively). The signals were fed into a differential amplifier to obtain the difference between the two pressures ($\Delta P = P_{inlet} - P_{outlet}$), which was plotted on a flatbed chart recorder (BD 41, Kipp en Zonen, Delft, The Netherlands). ΔP reflects the resistance of the tracheal segment to perfusion and is a function of the mean diameter of the trachea between the pressure taps. The transmural pressure in the trachea was set at 0 cm H_2O. At the perfusion flow rate used, a baseline ΔP of 0.1 to 1.0 cm H_2O was measured, depending of the diameter of the preparation. After a 45

min equilibration period with three washes with fresh KH (both IL and EL), 1 μM isoproterenol was added to the EL compartment for maximal smooth muscle relaxation to assess basal tone. After three washes during at least 30 min, the trachea was exposed to EL 40 mM KCl in KH to obtain a receptor-independent reference response. Subsequently, the preparation was washed 4 times with KH during 45 min until basal tone was reached and a cumulative concentration response curve was made with IL methacholine. When used, heparin (from porcine intestinal mucosa; 250 u ml^{-1}) was applied to both the IL and EL reservoir, while L-NAME (0.1 or 1.0 mM) was applied to the IL reservoir, both 40 min prior to agonist-addition.

Bronchoalveolar lavage

Animals were anaesthetised with 20 mg ml^{-1} Brietal-sodium, 35 mg kg^{-1} ketamine hydrochloride and 6 mg kg^{-1} Rompun i.p. which ensured a fast, deep anaesthesia. The lungs were gently lavaged via a tracheal canula with 5 ml of sterile saline at 37 °C, followed by three subsequent aliquots of 8 ml saline. The recovered samples were placed on ice, and centrifuged at 200 g for 10 minutes at 4 °C. The combined pellets were resuspended to a final volume of 1.0 ml in RPMI-1640 medium and total cell numbers were counted in a Bürker-Türk chamber. For cytological examination, cytospin-preparations were stained with May-Grünwald and Giemsa. A cell differentiation was performed by counting at least 400 cells in duplicate.

Eosinophil peroxidase assay

BAL cells were centrifuged and resuspended in Hanks balanced salt solution (HBSS) to final density of 2.5×10^6 cells ml^{-1} and incubated with medium for 30 min at 37 °C. Cell incubation was stopped by placing the samples on ice, followed by immediate centrifugation and subsequent decantation of the supernatant for measurement of eosinophil peroxidase (EPO) activity. After decantation the cells were lysed, centrifuged and the supernatant was collected to measure the remaining intracellular EPO content.

The EPO activity in cell supernatants and cell lysates was analysed according to the kinetic assay described by White *et al.* (1991), which is based on the oxidation of O-phenylenediamine (OPD) by EPO in the presence of hydrogen peroxide (H_2O_2). Substrate was made by dissolving 0.018 % H_2O_2 and 16 mM OPD in 100 mM Tris (hydroxymethyl)-aminomethane-HCl buffer, pH 8.0, containing 0.1 % Triton X-100, immediately prior to use. Horseradish peroxidase (HRP) in increasing concentrations was used as a reference. For the assay, 50 μl of supernatants obtained from either stimulated, non-stimulated or lysed cells or 50 μl of HRP solution were combined with 75 μl of substrate in a polystyrne 96-well microplate and placed into a thermoregulating microplate absorbance spectrophotometer (Thermomax, Molecular Devices, Menlo Park, Ca, USA) at 37°C. Absorbance at 490 nm was measured

every 15 sec for 30 min; the velocity of the reaction (as a measure of EPO activity) was calculated by interpolation between 20 successive points (5 min) utilising customised software (Softmax v2.01, Molecular Devices). All samples were assayed in quadruplicate.

Data analysis

To compensate for differences in baseline ΔP and in ΔP changes in response to contractile stimuli due to variation in resting internal diameter of the preparations used, IL responses of the tracheal tube preparations to methacholine were expressed as a percentage of the response induced by EL administration of 40 mM KCl. The contractile effect of 10 mM methacholine (highest concentration) was defined as E_{max} [1,2]. Using this E_{max}, the sensitivity to methacholine was evaluated as pEC_{50} (-log EC_{50}) value. EPO activity was expressed as a per cent of total amount of EPO (amount of EPO in the supernatant of stimulated cells + the amount present in the supernatant of the lysed cells). Results are expressed as means ± SEM. Statistical analysis was performed using the Student's t-test for unpaired observations. Differences were considered statistically significant at $P<0.05$.

Materials

Ovalbumin (grade III), aluminium hydroxide, (-)-isoprenaline hydrochloride, heparin sodium salt from porcine intestinal mucosa (grade IA), L-NAME, O-phenylenediamine dihydrochloride, horseradish peroxidase and May-Grünwald and Giemsa stain were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and methacholine chloride from Aldrich (Milwaukee, WI, USA). Brietal-sodium (methohexital) was purchased from Eli Lilly (Amsterdam, the Netherlands), Ketamine hydrochloride from Parke-Davis (Barcelona, Spain), Rompun (2-(2.6-xylidino)-5.6-dihydro-4H-1.3-thiazine-hydrochloride, methylparaben) from Bayer (Leverkusen, Germany), and RPMI-1640 medium and Hanks balanced salt solution (HBSS) from Gibco Life Technologies (Praisley, Schotland).

Results

In line with previous studies [1,2,18][Chapter 2], a 2.0-fold increase in airway responsiveness to methacholine (E_{max}) was observed in perfused tracheal preparations from ovalbumin-challenged guinea pigs obtained after the EAR, as compared with unchallenged controls ($P<0.001$), without an effect on the sensitivity (pEC_{50}) to the agonist (Figure 1a,b; Table 1). Preincubation with heparin (250 U ml^{-1}; IL and EL) completely normalized the observed AHR to methacholine in preparations from ovalbumin-challenged guinea pigs ($P<0.001$; Figure 1b, Table 1), while heparin did not affect the response to methacholine in unchallenged control airways (Figure 1a, Table 1). In both conditions, the pEC_{50} was not affected by heparin (Table 1).

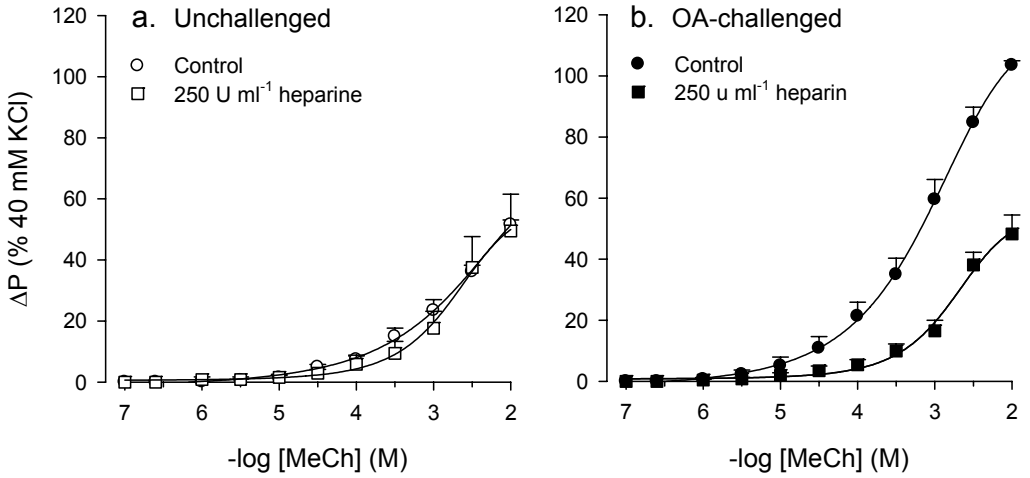

Figure 1: Methacholine (MeCh; IL)-induced constriction of intact perfused tracheal preparations obtained from (a) unchallenged and (b) ovalbumin (OA)-challenged guinea pigs, in the absence and presence of 250 u ml-1 heparine. Results are means ± SEM of 5-8 experiments.

In the presence of heparin, the normalized responsiveness of ovalbumin-challenged tracheae was reversed in a concentration-dependent fashion by coincubation with the NOS inhibitor L-NAME to the level of hyperresponsiveness of ovalbumin-challenged control airways after the EAR (Figure 2; Table 1).

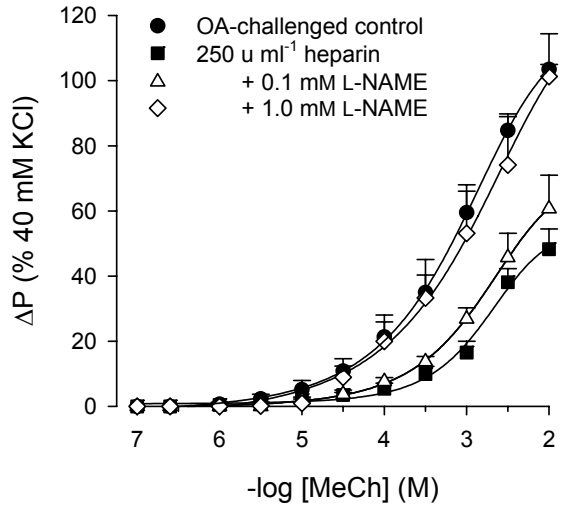

Figure 2: Methacholine (MeCh; IL)-induced constriction of intact perfused tracheae from ovalbumin (OA)-challenged guinea pigs, in the absence and presence of 250 u ml-1 heparin, alone or in combination with 0.1 or 1.0 mM L-NAME. Results are means ± s.e.m. of 4-8 experiments.

Table 1 Effects of heparin in the absence and presence of L-NAME on the responsiveness to methacholine of intact perfused tracheae from unchallenged and ovalbumin-challenged guinea pigs.

	Unchallenged			*Ovalbumin-challenged*		
	E_{max} (% KCl)	pEC_{50} (-log M)	*n*	E_{max} (% KCl)	pEC_{50} (-log M)	*n*
Control	51.6±1.5	2.94±0.14	5	103.4±1.5*	3.16±0.13	8
250 u ml^{-1} heparin	49.5±12.0	2.80±0.05	5	48.2±6.2†	2.98±0.14	5
+ 0.1 mM L-NAME	N.D.	N.D.		60.7±10.2†	2.95±0.06	4
+ 1.0 mM L-NAME	N.D.	N.D.		101.2±13.1‡	3.20±0.27	5

Results are means ± SEM of *n* experiments. N.D., not determined. **P*<0.001 compared with unchallenged; †*P*<0.001 compared with control; ‡*P*<0.01 compared with 250 u ml^{-1} heparin.

To establish eosinophil activation at 6 h after allergen challenge, release of EPO activity by BAL cells was measured. Figure 3 demonstrates that the spontaneous release of EPO by eosinophils obtained from ovalbumin-challenged animals was 5-fold enhanced compared to those obtained after saline challenge (*P*<0.001), indicating endogenous eosinophil activation at this time point. Also, the number of eosinophils in the BAL was significantly enhanced after allergen challenge (6.1 ± 1.0 x 10^6 *vs* 2.6 ± 0.5 x 10^6 in controls, *P*<0.05).

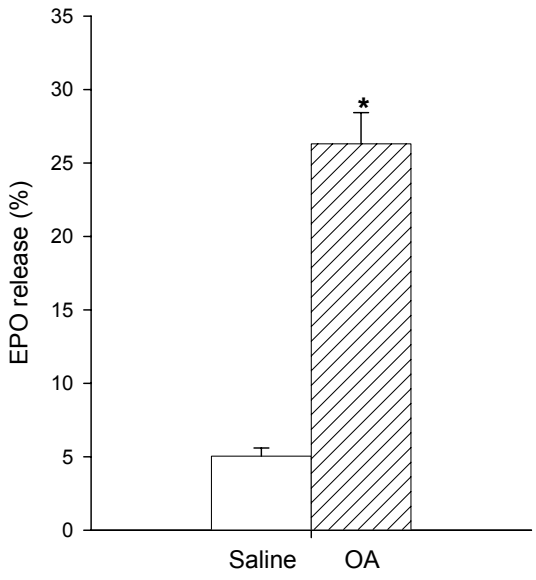

Figure 3: Spontaneous EPO release from BAL eosinophils obtained from sensitized guinea pigs at 6 h after saline or ovalbumin (OA) challenge, expressed as % of total cellular EPO content. 100 % represents 21.9 ± 5.2 and 22.5 ± 3.6 ng ml^{-1} EPO 10^6 cells^{-1} for saline and OA challenged animals, respectively. Results are means ± s.e.mean of 5 - 8 experiments. **P*<0.001 compared to saline.

Discussion

It has been demonstrated that MBP as well as its synthetic analogue poly-L-arginine can inhibit L-arginine transport through specific cationic amino acid transporters in rat alveolar macrophages and tracheal epithelial cells, thereby limiting NO synthesis in these cells [5]. In line with these observations, we have previously found that poly-L-arginine causes hyperresponsiveness to methacholine in perfused guinea pig tracheal preparations by causing a deficiency of agonist-induced NO [6]. Polycation-induced inhibition of L-arginine transport as well as NO deficiency and AHR were all inhibited by the polyanion heparin, indicating that the positive charge of the polycations may be important for these effects [5,6].

Previously, we have also observed that NO deficiency, caused by L-arginine limitation, is involved in the development of allergen-induced AHR after the EAR [1,2]. By using heparin, we did now find evidence that endogenous polycations, including eosinophil-derived MBP, could be involved in these processes. Thus, in a concentration which did not affect basal airway responsiveness to methacholine, heparin completely normalized the increased responsiveness in ovalbumin-challenged tracheae. This normalized airway responsiveness towards methacholine was dose-dependently reversed to hyperresponsiveness by the NOS inhibitor L-NAME, indicating that heparin had decreased the AHR by restoring the agonist-induced, cNOS-derived NO production. The maximal effect of L-NAME in the heparin-treated, ovalbumin-challenged preparations was similar to the maximal increase in responsiveness seen with L-NAME in unchallenged preparations [1,2,18,19][Chapter 2]. A possible explanation for the observation that a higher concentration of L-NAME (1.0 mM) was needed to fully inhibit NO production in the heparin-treated, ovalbumin-challenged airways than in control preparations (0.1 mM) [1,2,18,19][Chapter 2], is that heparin might scavenge L-NAME as well. Indeed, it has been demonstrated that heparin may bind monovalent cations [20].

The effect of heparin in reverting NO deficiency is presumably caused by preventing inhibition of L-arginine transport by endogenous polycations, including eosinophil-derived MBP. To ascertain that activation of eosinophil degranulation does indeed occur at 6 h after allergen challenge (after the EAR), we measured activity of EPO - one of the major polycationic and cytotoxic granule constituents of the eosinophil in addition to MBP - in the supernatant of isolated BAL cells. Indeed, the spontaneous release of EPO by eosinophils from allergen-challenged animals was considerably increased compared to controls, which corresponds with our previous finding of increased EPO activity in the (cell-free) BAL fluid at 6 h after ovalbumin challenge [7]. Moreover, the increased spontaneous EPO release of the isolated eosinophils *ex vivo* indicates sustained activation of these cells, and suggests that eosinophil-derived polycations and subsequent inhibition of L-arginine uptake will not disappear during equilibration of the perfused airways before heparin treatment.

Inhaled unfractionated or low-molecular-weight heparins have previously been demonstrated to inhibit allergen-induced early and late asthmatic reactions in allergic sheep [21,22] and guinea pigs [14] as well as exercise- and allergen-induced asthmatic reactions in asthmatic patients [23-25]. Moreover, heparin has been shown to inhibit AHR to methacholine, histamine and leukotriene D_4 in asthmatics [26,27] and to various cholinergic agonists in allergen-challenged sheep [21,28] and guinea pigs [14] *in vivo*. Restoration of bronchodilating NO production as indicated by our study is presumably involved. However, other non-anticoagulant, anti-inflammatory actions have also been implicated in the effects of heparin. Thus, in sensitized guinea pigs unfractionated and various modified heparins inhibited allergen-induced infiltration of eosinophils into the lung [29].

Low-molecular-weight-heparin is known to act as a competitive inhibitor of inositol 1,4,5-trisphosphate ($InsP_3$) receptors in mast cells and may thereby exert an inhibitory role on histamine release [30-32]. Indeed, the prevention of exercise-induced bronchoconstriction in patients with asthma was considered to be related to inhibition of the $InsP_3$-dependent stimulus-secretion coupling in mast cells [23,33]. Inhibition of $InsP_3$-induced Ca^{2+} release by heparin has also been found in (tracheal) smooth muscle cells [30,34]. However, since heparin did not at all affect methacholine responsiveness in our control preparations, a direct effect of the polyanion on airway smooth muscle Ca^{2+}-signaling and contraction can be excluded.

It has also been demonstrated that the polyanions heparin and poly-L-glutamate could recover neuronal autoinhibitory M2 muscarinic receptor function and vagally-induced AHR in antigen challenged guinea pigs, presumably by neutralizing eosinophil-derived MBP, which is an endogenous allosteric antagonist of the M2 receptor [35,36]. However, restoration of prejunctional M_2 receptor function cannot explain the action of heparin in our preparation, since only postjunctional M_3-muscarinic receptors were involved in the response of methacholine. In addition to reducing AHR, heparin is known to posses antiproliferative activity for airway smooth muscle cells [37]. Since airway remodelling, including increased airway smooth muscle mass, is involved in the pathology of asthma, the antiproliferative activity of heparin together with its ability to reduce AHR makes heparin of interest in the therapy of asthma.

Recently, we have demonstrated that another mechanism, involved in L-arginine limitation and subsequent NO deficiency and AHR, is increased activity of arginase, which hydrolyzes L-arginine to L-ornithine and urea and thus limits the L-arginine availability to NOS [18,38][Chapter 2]. Interestingly, like MBP and poly-L-arginine, the arginase product L-ornithine has also been demonstrated to inhibit L-arginine transport [39]. Thus, at least two mechanisms – increased arginase activity and inhibition of L-arginine uptake caused by endogenous polycations and possibly L-ornithine - are involved in the observed L-arginine limitation after the EAR. Since increasing the L-arginine availability by restoring L-arginine uptake after heparin

incubation normalizes the AHR after the EAR, it follows that arginase activity is becoming increasingly important in regulating the substrate availability for cNOS under conditions of substrate limitation. Therefore, restoration of the production of bronchodilating, cNOS-derived NO can be obtained by eliminating substrate competition by using an arginase inhibitor [18][Chapter 2] or increasing the L-arginine availability by restoration of the L-arginine uptake. It should be mentioned that a direct effect of heparin on NOS or arginase cannot explain the restoration of the AHR, since incubation of unchallenged control tracheae with heparin did not affect the responsiveness to methacholine.

In conclusion, using a guinea pig model of allergic asthma we have demonstrated that endogenous (eosinophil-derived) polycations may be importantly involved in allergen-induced NO deficiency and AHR after the EAR, presumably by inhibition of cellular L-arginine uptake through cationic amino acid transporters. Restoration of bronchodilating cNOS-derived NO production may be one of the mechanisms involved in the previously observed beneficial effects of heparin on AHR in allergic asthma.

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